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Fungal Allergens

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INTRODUCTION

Perspective of the Problem

Fungi grow almost everywhere, even as lichens inside Antarctic rocks (53). Fungi grow over a wide temperature range (−5 to 50°C and greater) (55, 89, 162), although individual species usually grow within a much narrower range. One of the most important physical parameters affecting fungal growth is moisture. Although it is widely stated that relative humidity over 70% is needed for active fungal growth, the water activity of the substrate is actually the critical parameter. Many species of fungi require high water activities, but the xerophilic (osmophilic) fungi are able to grow under lower water activity conditions than any other organisms (28, 55). Airborne spores are usually present in outdoor air throughout the year in high numbers and frequently exceed pollen concentrations by 100- to 1,000-fold (101, 105), depending on environmental factors such as water and nutrient availability, temperature, and wind. Most fungi commonly considered allergenic, such as *Alternaria* spp., *Cladosporium* spp., *Epicoccum nigrum*, *Fusarium* spp., or *Ganoderma* spp., display a seasonal spore release pattern, but this is less well defined than it is for pollens (20, 184).

Generally, indoor fungi are a mixture of those that have entered from outdoors (28, 101, 111) and those from indoor sources. *Aspergillus* spp. and *Penicillium* spp. are less common outdoors and are usually considered the major indoor fungi (20, 111). Studies have correlated outdoor spore counts with clinical symptoms (120), but much less is known about the effect of indoor spore concentrations (20, 160). Clearly, airborne spores are present in nearly all environments. These concentrations are frequently high enough to present a substantial antigen load to exposed individuals. The composite airborne spore load (i.e., the airspora) and the associated allergens remain incompletely characterized, especially in indoor air.

Biological Classification

Fungi are eukaryotic unicellular or multicellular organisms with absorptive nutrition and have been classified traditionally as members of the plant kingdom. More recently, a separate kingdom, Fungi (122, 216), was established for them, although species historically considered fungi are currently distributed among several kingdoms (18). Traditionally, fungal life cycles have been divided into perfect (sexual) and imperfect (asexual) states, depending upon the spore type produced. Current terminology refers to these states as the teleomorph and anamorph, respectively, which together are a holomorph. Conidium (plural, conidia) is a term used for an externally borne asexual spore produced by anamorphs of filamentous fungi. Some filamentous fungi predominantly or exclusively produce conidia rather than sexual spores. These have been referred to as Fungi Imperfecti or deuteromycetes but are termed anamorphic fungi in this review. Most of the species, including all those currently recognized as allergens, are ascomycetes. The classification of fungi covered in this review is presented in Table 1 (89). The species of fungi that produce airborne spores can be found within the divisions Dikaryomycota (ascomycetes and basidiomycetes), Zygomycota, and Oomycota (order Peronosporales).

Types of Medical Disorders Associated with Fungi

Fungi cause a number of infectious diseases. These range from superficial skin lesions primarily of cosmetic concern to potentially fatal systemic mycoses. Fungal infections are covered in numerous medical microbiology and specialty texts; they are beyond the scope of the present discussion, however, and will not be discussed further, although *Trichophyton* infections have been linked to asthma in some cases (43, 150).

Many fungi produce potent toxins. More than 200 toxins are

TABLE 1. Taxonomic distribution of selected genera of allergenic fungi[a]

TRUE FUNGI	
Phylum Zygomycota	
Class Zygomycetes	
Order Mucorales	*Mucor*, *Rhizopus*
Phylum Dikaryomycota	
Subphylum Ascomycotina	
Class Ascomycetes (including imperfect forms)	
Order Dothidiales	*Alternaria*, *Cladosporium*, *Epicoccum*, *Drechslera*, *Stemphylium*, *Wallemia*
Order Eurotiales	*Aspergillus*, *Penicillium*
Order Helotiales	*Botrytis*
Order Hypocreales	*Fusarium*
Order Onyngeales	*Trichophyton*
Class Saccharomycetes	*Saccharomyces*, *Candida*
Subphylum Basidiomycotina	
Class Holobasidiomycetes	
Order Agaricales	*Coprinus*, *Lentinus*, *Pleurotus*, *Psilocybe*
Order Aphyllophorales	*Ganoderma*, *Merulius*
Order Lycoperdales	*Calvatia*, *Geaster*
Class Phragmobasidiomycetes	*Dacrymyces*
Class Teliomycetes	
Order Uredinales	Rusts
Order Ustilaginales	Smuts, red yeasts (*Sporobolomyces*)
PROTISTAN FUNGI	
Phylum Oomycota	
Class Oomycetes	
Order Peronosporales	*Phytophthora*, *Plasmopara* (plant downy, or false, mildews)

[a] According to the systematic arrangement presented by Kendrick (89).

TABLE 2. Categories of hypersensitivity

Hypersensitivity type	General term	Agent	Mechanism	Example
I	Anaphylactic, allergic	IgE antibodies	Mediator release from IgE-sensitive mast cells or basophils	Asthma, hay fever
II	Cytotoxic	IgG or IgM antibodies	Antibodies and complement react on cell surfaces, cells lyse	Rh hemolytic disease
III	Immune complex induced	Antigen-antibody complex	Antigen-antibody complexes activate complement; mediator release from leukocytes	Serum sickness
IV	Cell mediated	T lymphocytes	T lymphocytes and macrophages reacting with antigens release mediators	Contact dermatitis, e.g., poison ivy

known (89), most of which have been identified from growth on plant materials, including those used for food. Probably the best known are the aflatoxins (potent carcinogens) produced by *Aspergillus flavus* and *Aspergillus parasiticus* (89). Fungal toxins are best known from ingestion exposure. These toxic reactions to fungi must be differentiated from allergic responses that may occur following ingestion of mushrooms or of food contaminated with fungi (102, 190). Allergic reactions to ingested fungi have been described infrequently, although one convincing case report of anaphylaxis due to mushroom consumption exists (190). Allergic reactions typically occur within 30 min of exposure, whereas toxic reactions may not appear until 6 to 8 h after ingestion. The longest delays between exposure and symptom onset are associated with the toxins that cause the most serious consequences. Toxic reactions are nonimmunologic; thus, toxic reactions can occur with the first encounter. Allergic reactions, on the other hand, require a previous, sensitizing exposure. Fortunately, most significant mycotoxins are of low molecular mass (<1 kDa) (89) and can be removed from fungal allergen extracts by dialysis.

Hypersensitivity has been characterized immunologically into four types of reactions on the basis of clinical presentation and the underlying mechanisms (36). The salient features are presented in Table 2. Type I (immediate) hypersensitivity (allergy) is triggered by the interaction of immunoglobulin E (IgE)-specific antigens, which are called allergens, with IgE on the surface of mast cells or basophils. Type II (cytotoxic) hypersensitivity occurs when antigens bound to cells are recognized by antigen-specific IgG or IgM. The surface-associated immune complexes are recognized by complement, and cell lysis occurs. Type III reactions are due to circulating immune complexes that attach to vessel walls and activate complement. Neutrophils phagocytize the complexes and induce a diffuse inflammation called vasculitis. Type IV (cell-mediated) reactions are mediated by antigen-specific T cells that release lymphokines in response to their encounter with antigen. A mononuclear cell infiltrate, which peaks at 24 to 72 h following exposure to antigen, occurs. As with other hypersensitivity reactions, this phenomenon requires previous exposure to the antigen. Many hypersensitivity conditions are combinations of these four types of reactions.

The term allergy in this review is applied to type I hypersensitivity. There is not, however, complete agreement about the definition of the term allergy. A recent authoritative treatment of indoor allergens by the Institute of Medicine defines allergy as the state of immune hypersensitivity that results from exposure to an allergen and is distinguished by overproduction of immune system components (151). Hypersensitivity pneumonitis and allergic bronchopulmonary aspergillosis (ABPA) are two respiratory disorders with type I hypersensitivity components, but neither syndrome is strictly an allergic phenomenon by our definition. These disorders are described in detail elsewhere (51, 57, 58), but they are discussed here briefly for comparison with type I hypersensitivity.

Hypersensitivity pneumonitis (or extrinsic allergic alveolitis) is an immunologic lung disorder caused by sensitization to various inhalant antigens such as fungi, actinomycetes, organic dusts, and certain highly reactive inorganic chemicals (51). The pathogenesis of hypersensitivity pneumonitis includes a mixture of immune complex (type III) and cell-mediated (delayed, type IV) hypersensitivity responses.

ABPA is an inflammatory disease in which fungi (usually *Aspergillus fumigatus*) grow in mucous secretions in the lung. ABPA is thought to involve type I and type III hypersensitivities associated with IgE- and IgG-mediated immune response to *Aspergillus* species (57). Several allergens of *Aspergillus fumigatus* have been characterized (90, 91, 100, 103, 130, 147, 211). As discussed later, the major allergen, Asp f 1, is homologous with mitogillin (the protein sequences are approximately 95% identical), a potent cytotoxin (10). Although allergens are involved in ABPA, there are other hypersensitivity components and nonimmunologic components of ABPA. ABPA appears to be more prevalent than was previously thought, and other genera of fungi are recognized to cause similar or identical diseases.

IgE-MEDIATED HYPERSENSITIVITY (ALLERGY) TO FUNGI

The term allergy was coined in 1906 by von Pirquet to describe an altered reactivity in living beings (i.e., an IgE-mediated hypersensitivity) caused by a foreign substance (208). Allergens, therefore, are the subset of antigens that stimulate an IgE-mediated response. Genetic factors are known to influence the ability to mount an IgE-mediated reaction (124), and those individuals with sustained elevated IgE levels are referred to as atopic. Type I allergic disease to fungal allergens is typically manifest either as rhinitis (hay fever) or as asthma. Allergic reactions, including respiratory allergy, may occur in two phases. The early-phase reaction occurs within minutes as a result of the release of preformed mediators. Late-phase responses occur 3 to 4 h after allergen exposure as a result of cellular infiltrates responding to early-phase mediators. A dual reaction involves both early- and late-phase reactions. Emerging evidence indicates that a significant, persistent inflammatory component in addition to IgE-triggered effects underlies the etiology of asthma.

Fungal allergy is not as well defined as other seasonal allergies and probably causes problems indoors for fewer people than do major allergens from cats, mites, or cockroaches. A significant portion of the atopic population does have underlying sensitivities to fungal spore allergens, although subjects

sensitized to a single fungal species are quite rare. In one study involving 6,000 patients with allergy, less than 1% were allergic to *Alternaria alternata* only, one of the species to which reactivity is most common (82). Thus, the common pattern is for subjects to react to many species. Furthermore, fungal allergies are more difficult to diagnose and treat than other allergies since fungi are far more numerous and antigenically variable than other allergens and are exceedingly difficult to avoid. Thus, management of fungal allergy can be a formidable clinical challenge.

Fungi as Aeroallergens

Fungal spores are universal atmospheric components indoors and outdoors and are now generally recognized as important causes of respiratory allergies. Allergic reactions associated with fungi involve the lower respiratory tract more frequently than do pollen allergies (105). Fungal spores (111) and spore extracts, when administered during provocative inhalation challenge tests, can cause immediate bronchoconstriction in sensitive subjects (62, 111, 117, 121). Reports of nonrespiratory symptoms, food allergy, or contact urticaria are anecdotal (190).

More than 80 genera of fungi have been associated with symptoms of respiratory tract allergy (28, 55, 104). Aerobiologic surveys show that fungal spores are present in the atmosphere worldwide. Multiple species may be observed at any time of the year, but in temperate climates, spore numbers peak during summer and fall, decrease with cooler temperatures, and are absent, at least outdoors, where snow cover occurs (3, 105, 185). Despite the clinical importance and abundant release of fungal spores, relatively few investigations have focused on the relationship between airborne spores and allergic disease.

Allergic reactions normally occur at the site of allergen deposition. Most inhaled particles of >10 μm (most pollens and some larger spores) are deposited in the nasopharynx and are associated with nasal and/or ocular symptoms generally referred to as hay fever (118). Conversely, particles of <10 μm, especially those of <5 μm, can penetrate the lower airways, where allergic reactions tend to manifest as asthma (40, 146). Fungal spores differ in size and are associated with both upper and lower respiratory symptoms. Additionally, there is now evidence that secondary dispersal of allergens, i.e., on other, smaller particles, possibly spore fragments, may serve as a vehicle for allergens. This would permit the deposition of allergens even from large spores such as *E. nigrum* into the lower airways.

Correlations between Airborne Prevalence and Clinical Relevance

Ascomycetes, basidiomycetes, and zygomycetes are the major fungal groups that contain genera known to produce allergens and elicit allergic reactions. Collectively, these groups contribute most of the spores found in air. Early sampling methods for airborne fungal particles had several biases. These methods were nonvolumetric, which tends to favor large spores or spores that disperse in clumps. Recovered fungi were usually detected by their growth on culture medium, which selects for fungi able to grow on the medium used. Analysis of the sample usually consisted of identifying the cultures on the basis of sporulation characters, which excludes species that do not reproduce in culture. Many anamorphic fungi are easily recovered by these methods, however, and most early research on fungal allergens centered on allergenic species of these.

As a result of more recent studies, in which nonviable, volumetric air sampling procedures have been used with direct microscopic analyses, it has been determined that conidia represent only 30 to 60% of airborne spores. The bulk of the remaining spores are ascospores and basidiospores. Recent evidence also indicates that the prevalence of hypersensitivity (skin test reactivity) to basidiospore and that to conidial allergens are also comparable (107). Thus, the intensity of exposure may indeed determine clinical relevance. The largest gap in our knowledge of aeroallergens pertains to ascospores, the sexual spores of ascomycetes. They constitute much of the balance of the airspora that is neither conidia nor basidiospores. Very little is known about ascospore allergens, although a certain degree of similarity to the allergens of conidial fungi might be inferred since the majority of the conidial fungi are anamorphs of ascomycete fungi.

Prevalence of Fungal Allergy

Allergic inhalant diseases such as asthma or rhinitis afflict 20% of the population in the United States and other industrialized countries, of which 10% have significant or severe allergic disease (54, 60, 136, 151, 214, 218). Skin test results further suggest that at least 3 to 10% of adults and children worldwide are affected by fungal allergy (20, 54, 131). The exact prevalence has not been established since reports of skin test reactivity to fungi range from 3 to 91%, depending upon the population studied, extracts used, and species tested (19, 63, 107, 166, 167, 187, 196). Prevalence of reactivity is dependent upon the source and batch of allergen as well as the selection criteria for the test subjects. For example, reactions to commercial *Cladosporium herbarum* extracts varied from 12 to 65% in the same population (2). Likewise, an experimental *E. nigrum* mycelium extract detected sensitivity in 70% of another population, whereas only 6% of the same group reacted to a commercial extract (152). Clearly, the true prevalence of fungal allergy will be unknown until standardized extracts are used to test well-defined populations.

Allergen and Allergen-Specific IgE Detection

Skin testing is the simplest method to detect IgE directed against a specific allergen. Two methods commonly used to diagnose allergies to inhalants, such as pollens, mites, or animal danders, are prick and intradermal skin testing. Skin prick testing correlates better with clinical history, radioallergosorbent test (RAST), and provocative inhalation challenge than the more sensitive intradermal test does (119). Intradermal tests also give false-positive results more frequently than prick testing does.

The RAST is an in vitro test designed to measure the circulating allergen-specific IgE antibodies of a patient. The RAST is generally considered less sensitive than skin testing, although a RAST conducted with partially purified fungal allergens can be comparable to skin tests in both sensitivity and specificity (8, 21, 96, 121). However, the use of exquisitely specific skin test reagents may indicate a lower prevalence of reactivity since less-specific reagents will cross-react with more species of fungi. Thus, cross-reactive antigens could simultaneously detect allergies to several different species with only a single skin test and provide a more robust screening reagent for fungal allergy.

Crossed immunoelectrophoresis (CIE) was used extensively for antigen analysis in the 1970s and 1980s, especially for complex mixtures such as allergen extracts. Crossed-line immunoelectrophoresis (CLIE) can identify common antigenic components in different sources and was used for early cross-

reactivity studies. Allergen identification is possible with crossed radioimmunoelectrophoresis (CRIE), a method in which a CIE agarose gel is overlaid with patient serum and any bound IgE is detected with radiolabeled anti-IgE. Although still useful to demonstrate antigenic purity, these methods have been replaced largely by immunoprinting (Western blotting [immunoblotting]) procedures.

The basophil histamine release test is another valuable in vitro assay, but it has limited clinical usefulness since it requires fresh leukocytes. False-negative results can occur, so the clinical symptoms of the patient must be considered when interpreting test results. Other methods of in vitro allergy testing, based on enzyme or chemiluminescent detection, are becoming commercially available and are generating much interest. None is as yet widely accepted.

Diagnosis of Fungal Allergy

Diagnosis of any allergic disease is based on the clinical symptoms of the patient, determination of exposure to the allergen, results of skin and in vitro tests (particularly the RAST), and sometimes, if necessary, provocative inhalation challenge testing (126, 142). Most subjects sensitive to fungi are also sensitive to other inhalant allergens, thus increasing the difficulty of diagnosis.

Allergic symptoms may occur in definite patterns that can provide clues to the diagnostician. Fungal allergic symptoms are not usually considered seasonal, however, as are pollen-related ailments. Moreover, many fungal spores derived from different species are similar morphologically; this can limit the completeness of spore identification in air sampling surveys.

Outdoor, indoor, and occupational exposures can all induce fungal sensitivity. Consequently, the variety of possibly allergenic fungi is far greater than can feasibly be included in any panel of skin test reagents. This emphasizes the value of environmental sampling, since the range of fungi to be considered can be narrowed effectively if an aerobiological identification is available. However, this would require sampling and analysis by certified personnel. On the basis of aerobiologic surveys conducted in different locations of the world, skin test studies, and fungal allergen characterization, the consensus is that skin test panels should include, at the minimum, *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *E. nigrum*, *Fusarium roseum*, and *Penicillium chrysogenum*. An optimal screening panel of fungal allergens is not currently available. It is also important to note that this list does not include basidiomycetes, primarily because suitable extracts are not commercially available.

Provocative challenge with specific fungal allergens can provide a definitive diagnosis of fungus-induced allergic disease. False-positive results can occur (186), however, so experienced investigators must perform and interpret these tests, which include nasal, conjunctival (127), or bronchial (142) provocation. Inhalation challenge can be an important tool in cases where conflicting or questionable data have been gathered, and it can confirm the clinical usefulness of allergen preparations in research settings as well. Conjunctival testing is less commonly used because of its poor reproducibility.

Despite the introduction of provocative challenge and the development of sophisticated in vitro tests, making the diagnosis of mold allergy remains difficult. Standardized extracts are not available, the average person is constantly exposed to a wide variety of propagules, and many subjects are also allergic to nonfungal inhalant allergens. Additionally, the symptoms may be due to a fungus either whose spores are not restricted to a given season or whose particular spore type is not tallied independently by a local pollen or spore station, if one exists. These factors would obscure any correlation between spore count and symptoms that might indicate the offending species. Development of standardized fungal extracts and more extensive and detailed reporting of pollen or spore counts would contribute significantly to the reliability of diagnoses of fungal allergies.

PREPARATION AND CHARACTERIZATION OF FUNGAL EXTRACTS

Progress in understanding fungal allergy has been impeded by the great variety of potentially allergenic fungi, the instability and variability of their allergens, lack of agreement on current fungal nomenclature, and the selection of adequate source material for the preparation of extracts. For these and other reasons, characterization of fungal allergens has lagged behind characterization of other aeroallergens. The exceptions are *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Alternaria alternata.*

Primary Difficulties with Fungal Allergens

Fungal spores are structurally very different from pollen, but commercial fungal extracts are usually prepared like pollen extracts. Fungal extracts generally contain proteins, carbohydrates, and proteolytic and glycosidic enzymes in various amounts (49). Carbohydrate material was long considered to be essentially nonallergenic, although reports have suggested the presence of carbohydrate allergens in *Alternaria alternata* (154). Recent, convincing evidence has been presented for IgE epitopes on fungal mannan preparations from the yeast *Malessezia furfur* (*Pityrosporum ovale*). However, most of the IgE-binding activity probably resides in the protein components of the fungal extracts (46).

Fungal allergen extracts are usually made from cultures. Fungal growth in culture is affected by temperature, moisture, light, and culture media. Conditions for optimal growth and sporulation may differ within and among species. Many fungi have inducible enzyme systems, and thus the composition of fungal media must be clearly defined for the consistent production of the desired enzymes or allergens (26). The inducible enzyme systems of fungi are especially significant since many of the allergens may, in fact, be enzymes such as enolase and alcohol dehydrogenase from yeasts (17, 52, 179). Strain variability within a given species presents an additional complicating factor. Even when a single strain is cultured under standard conditions, marked batch-to-batch differences may occur in protein composition and allergenic potency of extracts (26). Fungi also mutate readily in culture, contributing additional variables during in vitro cultivation (171). While the task is formidable, standardization of all phases of fungal extract production is an absolute requirement for significant progress in understanding fungal allergens.

Biochemical variability within a given species has been documented for several important allergenic fungi. *Candida albicans*, for example, is known to modulate cell wall composition during the growth cycle (156). Extracts from different strains of *Aspergillus fumigatus* (211), *Cladosporium herbarum* (204), *Alternaria alternata* (26, 189, 207), *E. nigrum* (45, 152), *Candida albicans* (171), and *Saccharomyces cerevisiae* (17) vary greatly in their compositions. Some researchers and commercial suppliers seek to overcome the problem of variability by culturing several isolates or growing different batches of the specific fungus and then pooling extracted material from each culture.

Variability of Commercial Extracts

In spite of the difficulties in standardization, crude extracts are used routinely to diagnose and treat fungal allergies in clinical practice. These extracts contain many undefined components, are extracted by a variety of procedures, and, consequently, may differ qualitatively and/or quantitatively. In fact, it is not unusual for a particular allergen extract to contain specific allergens that are entirely absent in other extracts of the same fungus produced by another manufacturer. The variability of the fungal strains contributes to this problem (28, 67, 207). Fungal isolates should thus be preserved in a manner that minimizes the need for serial subculturing. Cultures used for extraction should also be made generally available: either strains from established culture collections should be used or the strains used should be deposited in culture collections. Thus, the selection of a strain or a mixture of strains is crucial when preparing a representative extract. In addition, inconsistent allergenic activity among similarly or identically labeled commercial extracts has also been demonstrated in skin test and RAST studies (1, 12, 132, 220), and batch-to-batch variations have been documented (4, 26, 207, 220). The potency of fungal extracts prepared in-house for research is often significantly higher than that of commercial extracts (144). The availability of standardized extracts for testing and therapy is the greatest single need and yet the most recalcitrant problem in the treatment of fungal allergy today. The variability of commercial extracts is widely acknowledged, however, and considerable effort has been expended on improving, purifying, and standardizing extracts.

Extractions

Ideally, extraction procedures for inhalant allergens should simulate the manner in which the allergens are released under natural conditions. However, the fungal cell wall of some species can reduce and slow the discharge of allergens into solution (79). Release of significant amounts of allergen from spores with certain types of cell walls may require physical disruption or extended elution, neither of which is natural. Other spore walls apparently present minimal resistance to the release of allergenic proteins. Regardless of spore wall effects, potent extracts have been prepared from spores, culture medium, and fresh and dried mycelial mats. This extracted material is centrifuged or filtered, and the supernatant or filtrate frequently undergoes further processing, such as dialysis, to remove low-molecular-weight nonallergenic material, including some toxins. Several reagents, such as phosphate-buffered saline (PBS), sodium bicarbonate ($NaHCO_3$), distilled water, or Coca's solution (0.5% NaCl, 0.275% $NaHCO_3$), can successfully remove allergens from undisrupted conidia of some fungi, and shaking or stirring the suspension may increase release (102). Other spores, e.g., basidiospores, may require more rigorous extraction procedures. Cell disruption with glass beads in a cell homogenizer or blending in carbonate buffer provides greater allergen yields than passive methods alone, but complete cell disruption may increase the yield of nonallergenic proteins, effectively diluting allergens in the extract. Disruption may also increase the release of proteases that may degrade some allergens, although the proteases themselves are also potential allergens. Some allergens, notably Asp f 1, are only expressed, however, in actively growing cells (9). The standardization of extracts for Asp f 1 must, therefore, take growth conditions into consideration very carefully. An ideal extraction procedure has yet to be established. Studies with monoclonal antibodies (MAbs) would prove useful in establishing the release kinetics of the major allergen(s).

The release of allergens from *Cladosporium cladosporioides* is affected by the culture medium, extraction time, and spore (conidium) concentration (22). The greatest yields were obtained when the culture had a low spore concentration (0.1 mg [dry weight] per ml) or was subjected to a brief ultrasonication, but the yield varied little with the type of extraction solution used, including 0.1 N NaOH, Coca's solution, 50 mM Tris, or 0.9% NaCl. Mycelia of *Wallemia sebi* and two *Aspergillus* spp. were most active in the RAST within 20 min after homogenization in PBS (159). The yield of *Alternaria alternata* allergen from mycelium, as identified by CRIE, decreased when extraction continued beyond 6 h (195). Allergen yield from *Alternaria alternata* spores and mycelium was also greater when protease inhibitors such as phenylmethylsulfonyl fluoride or EDTA were included in the extraction buffer (145). Polyvinylpyrrolidone also aided extraction, presumably by binding polyphenols such as melanin and preventing coprecipitation of proteins (145). Polyvinylpyrrolidone would thus be most useful for fungi which are heavily pigmented with melanin, such as members of the Dematiaceae.

Many different procedures and buffers have been used to extract allergens from fungi, and no standard procedure appears appropriate for all species. Factors known to be important and which should be optimized include the extraction buffer, length of extraction time, whether to physically or sonically disrupt the cells, and whether to add preservatives, protease inhibitors, or polyvinylpyrrolidone. These must be determined empirically for each species.

Stability

Extracts must remain stable to be useful clinically or for research. As with all proteinaceous mixtures, the stability of allergenic extracts depends on the type and quantity of allergen, the storage temperature, and the presence of preservative and other (nonallergenic) material in the mixture.

At present, lyophilization is the best method to maintain the allergenic potency of fungal extracts (213). However, lyophilization is not an entirely innocuous procedure, and certain allergens may be permanently altered and/or rendered insoluble. Fractionation of lyophilized extracts may remove autolytic enzymes and further extend stability. Exposure to moisture and warmth will increase the degradation process; therefore, extracts must be desiccated and maintained at a cool temperature. Lyophilized *Alternaria alternata* extracts, for example, stored at −70, −20, and 4°C were all stable for at least 21 months as measured by RAST inhibition (68). Similarly, the allergenicity of native Cla h 2 (antigen-54 [Ag-54]) from *Cladosporium herbarum* was unaltered for at least 5 years when it was stored at −20°C, although deglycosylated Cla h 2 lost most allergenic activity in 2 years (192). Interestingly, both the native Cla h 2 and the deglycosylated allergen were stable when heated to 100°C. Stability studies of allergens from the basidiomycete *Calvatia cyathiformis* indicated that neutral and basic allergens lost activity more rapidly than acidic allergens did (77). This suggests that loss of potency of any given extract may be due to degradation of specific allergens rather than a general reduction in activity of all allergens. Factors affecting stability must be clearly identified for each type of allergenic extract to ensure longevity of the extracts.

All extracts must be kept sterile to protect the individuals tested and prevent microbial degradation of the extract. Moreover, reconstituted extracts must contain a stabilizer to prevent a rapid loss of potency; loss rates depend upon extent of the dilution. Stabilizers such as human serum albumin, glycerol, phenol, or ε-aminocaproic acid can preserve the integrity of

TABLE 3. Purified fungal allergens

Fungal species	Nomenclature		Molecular mass (kDa)	Isoelectric point (pI)	Reference
	Standard	Provisional/alternate			
Alternaria alternata		Alt-1	31	4.3	221
	Alt a 1[a,b]		28		125
		Alt a 29K[a,b]	29	4.2–4.6	38
	Alt a 1_{1563}[b]		31	4.0–4.5	143
		Alt a Bd29K[b]	29	4.2	42
		gp 70[c]	70	3.5	153
		Not yet designated[c]	60–66		29
		Basic peptide	31	9.5–9.8	25
Aspergillus fumigatus	Asp f 1	Ag 3	18		103
		Ag 20 kDa	20	5–6	165
Cladosporium herbarum	Cla h 1	Ag 32	13	3.4–4.4	13
	Cla h 2	Ag 54	23	5.0	13
Trichophyton tonsurans	Tri t 1		30		43
Calvatia cyathiformis		Cal c Bd9.3	16	9.3	74
Psilocybe cubensis		Psi c Bd48kD	48		158
	Psi c 2	Cyclophilin	16		81
Saccharomyces cerevisiae		Enolase	51		17
Candida albicans		Enolase	48		52
		Alcohol dehydrogenase	40		179

[a] Amino-terminal amino acid sequences agree for the allergens described by Matthiesen et al. (125) and Curran et al. (38).
[b] The allergens described by Matthiesen et al. (125), Curran et al. (38), Paris et al. (143), and Deards and Montague (42) are apparently the same allergens.
[c] The allergens partially characterized by Portnoy et al. (153) and by Bush and Sanchez (29) are recognized by MAbs raised against the corresponding allergen.

allergenic extracts (213). Fifty percent glycerol is probably the most effective stabilizer but is a strong irritant when used in reagents for intradermal testing. It is also very difficult to ensure that products used as stabilizers are completely innocuous for humans. Although ε-aminocaproic acid and dilute phenol are widely used, better stabilizers as well as effective protease inhibitors that are nontoxic and can be used in vivo are needed (49).

Source Material

It is presumed that individuals are exposed principally to spores or conidia rather than mycelium, and it is axiomatic that the best fungal extracts are derived from material that contains the most complete complement of relevant spore and/or mycelial allergens. In fact, several studies have identified allergens that are spore specific, which implies the need for spores as a source material for allergenic extracts from these species (15, 72, 215). Furthermore, some allergens are expressed only subsequent to germination (9, 110, 114), which necessitates the extraction of actively growing mycelium and metabolite mixtures (culture medium) containing metabolites from these species. Thus, the appropriate source material may require independent assessment by species.

Although spores may contain the most relevant fungal allergens, they can be difficult to obtain, and most commercial extracts are prepared from mycelium and contain few or no spores (71). This situation persists even with fungi for which sporulating cultures are readily produced. However, extracts of spore-free fungal material, such as mycelium, produce positive RAST reactions when tested with sera from subjects with fungal hypersensitivity (66, 72, 115, 144, 210, 215). A recent comparison of *Alternaria alternata* spore and mycelium extracts found that mycelium extracts have equivalent or greater potency than that of spore extracts on the basis of skin prick test, RAST inhibition, and basophil histamine release (50). In the basidiomycetes, spore and mycelial extracts of *Pleurotus ostreatus* were comparable in skin test activity, although some allergens were spore specific (215). Other fungal tissues, such as cap or stalk of basidiomycetes (mushrooms), are also more abundant than spores and deserve consideration as material for extraction. Spores may vary less from batch to batch than mycelium, but data are lacking. Mycelium, produced in culture, provides a readily available source for large quantities of extract. Mycelia are available independent of season and can be produced under standard conditions, and verification of identity is required only when the fungus is entered into the culture collection rather than after each collection from nature. At present, since there is no accepted standard source material for fungal allergen extracts, many researchers feel that spores should be included to ensure that all allergens are present.

New Allergen Nomenclature

As the number of identified allergens has increased, the number of different and often confusing terms for allergens (and sometimes for the same allergen) has also increased. To resolve this problem, the International Union of Immunologic Societies Subcommittee for Allergen Nomenclature presented a unified nomenclature system for highly purified allergens and individual components detected within complex allergen extracts (123). Allergen identification is based on multiple immunochemical and physicochemical techniques. The allergen source is clearly defined by fungal species and its purity. Purified allergens are designated by the first three letters of the genus, the first letter of the species, and a numeral for their order of isolation. Although roman numerals were originally used, those are now reserved for gene designations, and arabic numerals are used for protein designations (92). Fungal allergens are further labeled by the strain number. A summary of the fungal allergens defined to date is shown in Table 3.

REVIEW OF FUNGAL ALLERGEN STUDIES

Relatively few allergens of the fungi known to produce clinically relevant allergens have been characterized. The present discussion is restricted to species whose allergens have undergone some degree of biochemical or immunochemical characterization, although numerous other fungi are allergenic.

TABLE 4. Known anamorph or teleomorph (imperfect or perfect state) connections of selected allergenic ascomycetes[a]

Anamorph genus and/or species	Teleomorph genus	Order
Alternaria	*Clathrospora*	Dothidiales[b]
	Leptosphaeria	Dothidiales
	Pleospora	Dothidiales
Helminthosporium	*Pseudocochliobolus*	Dothidiales
Drechslera	*Pyrenophora*	Dothidiales
Curvularia	*Pseudocochliobolus*	Dothidiales
Stemphylium	*Pleospora*	Dothidiales
Ulocladium[c]	*Lasiobotrys*	Dothidiales
Penicillium	*Talaromyces*	Eurotiales
	Eupenicillium	Eurotiales
	Trichocoma	Eurotiales
Aspergillus	*Eurotium*	Eurotiales
	Dichlaena	Eurotiales
	Emericella	Eurotiales
	Edyuillia	Eurotiales
	Neosartorya	Eurotiales
	Petromyces	Eurotiales
Spondylocladium (*Stachylidium*)	*Nectriopsis*	Hypocreales
Fusarium	*Gibberella*	Hypocreales
	Nectria	Hypocreales
	Calonectria	Hypocreales
Botrytis cinerea	*Botryotinia*	Helotiales
Wallemia sebi	Unk[d]	Unk
Epicoccum nigrum	Unk	Unk

[a] Compiled from listings by Carmichael et al. (33). Anamorph indicates the asexual (imperfect) state of a fungus, teleomorph refers to the sexual (perfect) state, and the two states together constitute the holomorph of the fungus.
[b] Dothidiales herein refers to bitunicate ascomycetes.
[c] A distinction from *Alternaria* sp. is not clear (33).
[d] Unk, unknown.

Anamorphic Fungi

The common allergenic molds are, for the most part, anamorphs of ascomycetes. A selected list of fungal anamorphs commonly considered to contain important allergens and their related teleomorphs in the ascomycetes are presented in Table 4.

***Alternaria alternata*.** *Alternaria alternata* allergens have long been considered to cause significant respiratory allergies in patients in the United States and were implicated recently in serious cases of respiratory arrest (137). These arrests occurred among *Alternaria alternata*-sensitive children and young adults and were temporally associated with observed high levels of *Alternaria alternata* spores in outdoor air during summer and fall months. Although this association has not been confirmed elsewhere, the implication that fungal allergy contributes to critical attacks of respiratory arrest greatly increases the clinical importance and need for proper diagnosis of fungal allergy.

Although other *Alternaria* species are probably relevant clinically, most research has been directed towards *Alternaria alternata* (206). Among the first allergens to be defined was a partially purified mycelial allergen isolated by gel chromatography. This glycoprotein fraction was called Alt-1, had an apparent molecular mass of between 25 and 50 kDa, and contained at least five isoelectric variants between pI 4.0 and 4.3 (221). Ag-1 and Ag-8 are variants of this antigen (134, 189).

Subsequently, several groups isolated the major allergenic component. Two groups of investigators used anion-exchange chromatography to purify Alt a 1 (note new nomenclature) from mycelium (42, 143). Paris and coworkers (143) designated the allergen Alt a 1_{1596} (Alt a I_{1596}; 31 kDa; pI 4.0 to 4.5) and determined it to be a heat-stable glycoprotein containing 20% carbohydrate which is located in the cytoplasm of both mycelium and spores (145). Deards and Montague designated this allergen Alt a Bd29K (pI 4.2; 29 kDa) and determined that it is composed of 15-kDa subunits (42). Two other research groups have established that the reduced form of Alt a 1 has 12- and 15-kDa components, and the N-terminal amino acid sequence of the 12-kDa component has been determined (38, 125). Another purified allergen, gp70, is a glycoprotein (70 kDa; pI 3.3) that constitutes 13% of the dry weight of the *Alternaria alternata* extract and may represent a dimer of Alt a 1 (153). Of 16 subjects who tested positive in skin tests with *Alternaria alternata* extracts, 11 reacted to gp70, although the purified allergen was less potent than the crude extract in these skin tests. Bush and Sanchez determined the amino acid composition of a 60-kDa *Alternaria alternata* allergen and established the partial cDNA sequence for another *Alternaria alternata* allergen (29). Another partially purified allergen that has been described as a basic peptide (pI 9.5; 6 kDa) is able to induce a wheal-and-flare skin reaction in sensitized subjects (25). Eighteen of 20 (90%) atopic, *Alternaria alternata* skin-test-positive subjects in this study reacted to this basic peptide, which was designated Alt a 2 d (Alt a II d) (72).

Tremendous recent advances in the molecular characterization of *Alternaria alternata* allergens have been made. Direct comparison of the isolated Alt a 1 variants from different laboratories is essential to fully elucidate the molecular structure of these allergens and to identify the specific epitopes involved in allergy. In a recent collaborative study of source strains, four research groups examined 12 isolates. These isolates were assigned to four strain groups on the basis of their morphology, enzyme profiles, and allergen content (155). Breitenbach and coworkers have reported nucleotide sequences of three cDNA clones coding for 53-, 22-, and 11-kDa allergens (23). All of these allergens are homologous to *Cladosporium herbarum* allergens and are abundant, cytosolic housekeeping proteins. As more sequences become available, the common identity of the allergens isolated by different laboratories (Table 3) can be established.

***Aspergillus* spp.** *Aspergillus* sp. antigens have also been studied thoroughly because *Aspergillus* spores were among the first to be recognized as important aeroallergens, and these fungi cause several other diseases. Reviews of *Aspergillus* sp. antigens and allergens were presented recently (99, 114).

Two antigens (18 and 20 kDa) have been purified from *Aspergillus fumigatus* by chromatography (103, 165). The 18-kDa antigen was isolated from the mycelium of 10 different strains of *Aspergillus fumigatus*. Monospecific polyclonal rabbit antiserum directed against this antigen reacted with antigens of 26, 28, 44, and 46 kDa as well (103). The characteristics of the 18-kDa antigen suggest that it is similar to Ag-3 or Ag-10 described earlier (114) from CIE patterns. The 20-kDa allergen is a glycoprotein (pI 5 to 6) that does not bind concanavalin A and appears to be different from antigens Ag-3, Ag-5, Ag-7, and Ag-13. Another glycoprotein allergen, designated gp55, was recently isolated; its allergenic activity was sensitive to protease but not to deglycosylation (199). On the basis of the amino-terminal protein sequence, this is a novel protein.

The amino acid sequence of the 18-kDa protein (Asp f 1 [Asp f I]) has been determined partially (9, 10). Asp f 1 mRNA was detected in *Aspergillus fumigatus* but not in seven other *Aspergillus* spp. Asp f 1 shows extensive sequence homology (95%) to mitogillin (a cytotoxin) produced by *Aspergillus restrictus* (9). A cross-inhibition radioimmunoassay with a murine MAb and human IgG and IgE antibodies revealed that Asp f 1 and mitogillin are indistinguishable antigenically (10). The simultaneous toxicity and allergenicity of Asp f 1 have

significant implications for understanding the etiology of ABPA. Asp f 1 cDNA from *Aspergillus fumigatus* has now been cloned, and recombinant allergen has been expressed and used in skin test trials (129, 130). A recombinant *Aspergillus fumigatus* protein (65 kDa) expressed from a cDNA clone has also been shown to bind IgE from ABPA patients (98). On the basis of cDNA and deduced amino acid sequence homology to hsp 90 from other organisms, this allergen is apparently a heat shock protein of the hsp 90 family. The presence of nonallergenic homologs provides a significant opportunity to study the basis of allergen epitopes by focusing on the differences between these proteins, i.e., hsp 90 from *Aspergillus fumigatus* (allergenic) and hsp 90 from humans (nonallergenic). These important achievements provide a significant aid to standardizing *Aspergillus* sp. extracts and diagnosing *Aspergillus*-related diseases. Perhaps more significant, structural analysis of a fungal allergen by recombinant DNA technology is proceeding.

Several enzymes from *Aspergillus* spp. are used as conditioners or enhancers in bakery products. Allergenic activity among baking and food industry workers has been demonstrated for cellulase from *Aspergillus niger* and α-amylase from *Aspergillus oryzae* (157). These enzymes also partially inhibited the allergenic activity of both *Aspergillus niger* and *Aspergillus oryzae* extracts.

***Cladosporium* spp.** Spores of *Cladosporium* spp. probably occur more abundantly worldwide than any other spore type and are the dominant airborne spores in many areas, especially in temperate climates (184). Although *Cladosporium cladosporioides* can be the most prevalent airborne species (101), *Cladosporium herbarum* frequently dominates the outdoor airspora and its mycelial allergens have been studied very intensively. At least 60 antigens derived from *Cladosporium herbarum* were detected by CIE, and 36 were allergenic by CRIE (11). Two major *Cladosporium herbarum* allergens have been purified and characterized (13). Cla h 1 (Cla h I or Ag-32) is a small (13-kDa) acidic allergen composed of five isoallergens (pI 3.4 to 4.4). Cla h 2 (Cla h II or Ag-54) is a slightly larger (20- to 22-kDa), less acidic (pI 5.0) glycoprotein containing 80% carbohydrate (mannose-galactose-glucose, 1:0.6:1.3) (191–194). After carbohydrate and protein moieties were separated, only the protein retained IgE-binding properties; interestingly, this binding was stronger than that for native Cla h 2. Four cDNA clones from *Cladosporium herbarum* that code for allergens were isolated recently (23). Sequence homology indicated that these allergens are aldehyde dehydrogenase (Cla h 3; 53 kDa), P2 ribosomal protein (Cla h 4; 11 kDa), YCP4 yeast protein (Cla h 5; 22 kDa), and enolase (Cla h 6; 48 kDa). These well-characterized allergens should prove extremely useful as models for allergen analysis of this ubiquitous fungus spore type.

***Botrytis cinerea*.** *B. cinerea* is a widespread fungus. In Europe, maximal spore counts occur during late summer. Prevalence of skin test reactivity, i.e., immediate wheal and flare, to *B. cinerea* in Europe is comparable to that of *Aspergillus* spp. and greater than that of *Cladosporium* and *Penicillium* spp. With an extract of a mixture of *B. cinerea* spores and mycelium in a Western blot procedure, IgE antibodies specific for *B. cinerea* allergens could be demonstrated in the sera of sensitized subjects (86). A mixture derived from mycelia and culture fluid of *B. cinerea* harvested at different growth phases was evaluated for binding of anti-*B. cinerea* IgE and IgG in a pool of patient sera. IgG and IgE binding peaked during the early phase of growth (88). Since the prevalence of *B. cinerea* spores and skin test reactivity is significant, further characterization of these allergens is warranted.

***Epicoccum nigrum*.** *E. nigrum* , the only species in this genus, is reported frequently in air samplings (110). To date, studies indicate skin test and RAST reactivity to *E. nigrum* ranging from 20 to 30% in atopic subjects (45, 69, 152). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblots have revealed several IgE-binding bands, usually from 8 to 110 kDa, in both spore and mycelium extracts; 13 allergens were common to both, 9 were in the spore extracts only, and 6 were unique to the mycelial extract (45). Thus, the most representative *E. nigrum* extracts would be those prepared from mixtures of spores and mycelium.

***Fusarium* spp.** Airborne spores of *Fusarium* spp. are widespread but especially common in agricultural areas (128, 209). Approximately 24% of atopic individuals in one study were skin test and RAST positive to *Fusarium solani* extracts (140). Thirty-eight antigens have been detected by CIE, and 21 of these are recognized as allergens by CRIE. Gel filtration fractionation of an *F. solani* extract resolved three UV-absorbing peaks (>341, 341, and <42 kDa) which bound IgE. RAST inhibition was greatest for the >341-kDa fraction (158).

Extracts of *F. solani* culture filtrate, mycelium, and spores were analyzed by blotting each against a panel of 15 serum samples from asthmatics who were skin test (intradermal) reactive to *F. solani* (201). A collective total of 23 allergens were recognized among the three sources by use of this panel. A 65-kDa allergen bound IgE from 100% of the test sera, and the culture filtrate contained 18 allergens, the greatest number among the *F. solani* sources. *F. solani* culture filtrate contained more allergens than filtrates of *Fusarium equiseti* or *Fusarium moniliforme* did (18, 12, and 9, respectively) (202). Of the 18 allergens of *F. solani*, 14 shared epitopes with either *F. equiseti* or *F. moniliforme*, as indicated by immunoblot inhibitions (202). The presence of shared epitopes among different *Fusarium* spp. and the prevalence of exposure to these spores suggest that further characterization of *Fusarium* allergens is warranted.

***Helminthosporium* spp.** *Helminthosporium* spp. previously included a large number of species with similar conidium morphologies. The genus is now defined in a more narrow sense and contains relatively few species. Thus, most previous work with allergy to *Helminthosporium* spp. probably used fungi that are now included in the genera *Drechslera*, *Exserohilum*, and *Bipolaris*. Taxonomy aside, the prevalence of skin test reactivity to members of this group is significant (93, 96, 167). Although *Helminthosporium* spp. are frequently included in skin test surveys, only a preliminary characterization of the allergens has been reported. Spore and mycelium extract of *Helminthosporium halodes* is a complex mixture of at least 17 allergens, ranging from 14 to 94 kDa, which bind IgE in SDS-PAGE IgE immunoblots (86). Minimal cross-reactivity was seen between extracts of *H. halodes* and either *Alternaria alternata* or *B. cinerea*. Further characterization of the allergens of these fungi should be a research priority.

***Penicillium* spp.** Spores of *Penicillium* spp. do occur outdoors but are far more important as indoor fungal contaminants (27). Bronchial challenges with *Penicillium* sp. spores induced immediate- and delayed-type asthma in sensitized subjects (111). SDS-PAGE immunoblots of *Penicillium notatum* extracts detected 11 IgE-binding proteins from 20 to 90 kDa (178). The most important were the 68- and 64-kDa allergens which reacted with 56 and 46% of 39 individual serum samples, respectively. MAbs against the 68-kDa allergen (isoelectric point, 6.0 to 6.1) cross-reacted with a 67-kDa band from two (of 10) other *Penicillium* spp. and from *Aspergillus fumigatus* (177). Other allergens have not been characterized.

***Trichophyton* spp.** *Trichophyton* spp. classically induce delayed-type or cell-mediated hypersensitivity. The possible role

of *Trichophyton* spp. in IgE-mediated urticaria, asthma, and rhinitis has been debated since 1930, but the relevance of these species remains controversial. IgE antibodies to *Trichophyton tonsurans* have been found in skin-test-positive subjects (150). Subjects with chronic skin infection and intrinsic asthma developed an immediate hypersensitivity response when tested by bronchial provocation or by double-blinded nasal challenge (212). A 30-kDa, hydrophobic major allergen of *Trichophyton tonsurans* (Tri t 1) was purified by gel filtration and hydrophobic interaction chromatography, and the sequence for 30 N-terminal amino acids was determined (43). Two MAbs that recognize distinct epitopes on Tri t 1 were prepared. Although no data are available currently, studies with these antibodies should help determine the importance of *Trichophyton* spp. as an allergen (43).

Basidiomycetes

Basidiomycetes are physically the largest and most morphologically complex fungi. There are an estimated 20,000 to 25,000 species, including mushrooms, puffballs, bracket fungi, rusts, and smuts. Two case reports, one in the 1920s and another in the 1930s, indicated that smut chlamydospores (32, 217) and rust urediospores contained allergens. Both spore types are asexual. Although widely used for decades in skin testing, rust and smut extracts have not been characterized. Most basidiomycete allergen studies to date have focused on the sexual spores, i.e., basidiospores, of mushrooms, bracket fungi, and puffballs.

Basidiospores occur in the air in high concentrations in many parts of the world, and positive skin tests (64, 70, 107, 166), RASTs (34), and bronchial reactivity (113) have been detected in hypersensitive subjects. Gregory and Hirst reported in 1952 that basidiospores provoke respiratory symptoms (59). Twenty years later, Salvaggio and coworkers associated increased levels of small spores (including basidiospores) in outdoor air with epidemic asthma in New Orleans, La. (163). These two papers provided a significant impetus for workers to characterize basidiomycete allergens. To date, some 50 species have been tested for allergenicity, and about 25 species have been determined to be allergenic (28). Also, at least three basidiomycetes, *Lentinus edodes* (shiitake), *Pleurotus ostreatus*, and *Merulius lacrymans*, have been shown to cause hypersensitivity pneumonitis (24, 28, 132, 133, 135, 173). Inhalation challenges with shiitake mushroom spores have confirmed the allergenicity of the spores (173). Allergens have been described in some detail for five genera of basidiomycetes and are discussed below.

***Calvatia* spp.** *Calvatia* spp. are seasonally occurring puffballs that produce tremendous numbers of spores. Isoelectric focusing analysis of *Calvatia cyathiformis* has revealed 21 allergens. Immunoprints of crude and fractionated extracts have indicated that two allergens (pI 9.3 and 6.6) reacted with 68 and 63%, respectively, of serum samples from 19 patients who were skin test positive to *Calvatia cyathiformis* antigen (76). These were provisionally designated Cal c Bd 9.3 and Cal c Bd 6.6. Purification attempts indicated that both allergens had limited stability, but the protein of pI 9.3 was particularly labile (77). Preparative isoelectric focusing was used to isolate the allergen of pI 9.3 which has a molecular mass of 16 kDa (80). A comparison of four *Calvatia* species indicated a significant degree of allergenic cross-reactivity among three species; only *Calvatia gigantea* was not cross-reactive (109). The rate of allergen release has also been studied in *Calvatia cyathiformis*. When compared with the thin-walled spores of *Pleurotus ostreatus*, *Calvatia cyathiformis* spores released less allergen and released the allergen more slowly (79). The clinical significance of different allergen release patterns among different species remains to be established.

***Pleurotus ostreatus* (oyster mushroom).** *Pleurotus ostreatus* has long been known to be antigenic in experimental animals and allergenic in humans (31, 107, 116). *Pleurotus ostreatus* occurs widely; thus, nonoccupational exposure is likely. Since this is a cultivated mushroom, however, there are also significant occupational exposures. Oyster mushroom spores contain potent allergens capable of inducing bronchoconstriction, and inhalation of the spores can result in hypersensitivity pneumonitis, especially among mushroom workers (37, 113).

Oyster mushroom allergens have a broad range of physicochemical characteristics and occur in several sources. An extract of *Pleurotus ostreatus* mycelium had 33 proteins to which specific antibody could be detected. Thirteen of these were identified as allergens by CRIE (105). In another study, *Pleurotus ostreatus* spore, mycelium, and cap extracts induced antibodies in rabbits against 27, 16, and 17 proteins, respectively (215). Five of 27 antigens in spores were allergens. In a later study, gel filtration and hydrophobic interaction chromatography were used to isolate the RAST-active fraction of *Pleurotus ostreatus* spores. Isoelectric focusing immunoprints demonstrated IgE-binding bands in the basic (pI 9.0 to 9.3) and acidic (pI 3.5 to 4.5) regions (78). These allergens, like those of *L. edodes*, are released rapidly from intact spores, in contrast to the allergens of *Psilocybe cubensis* or *Calvatia cyathiformis* (79). The allergen release characteristics may be related to spore wall properties.

***Coprinus* spp. (inky caps).** Allergen activity of *Coprinus quadrifidus* spores and *Coprinus commatus* mycelium extracts has been demonstrated by skin test and RAST (107, 116). Spores, cap, and stalk extracts of *Coprinus quadrifidus* were fractionated by gel filtration (41). Comparably sized components of each extract (approximately 10.5 to 25 kDa) had the highest RAST activity. RAST inhibition using all three *Coprinus quadrifidus* extracts indicates that important epitopes were common to all three. *Coprinus* spp. are common and widely distributed in lawns and gardens and thus are likely sources of basidiospore exposure.

***Ganoderma* spp.** *Ganoderma* spp. are important wood decay fungi that produce large shelf-like fruiting bodies called brackets or conks. *Ganoderma* sp. spores occur widely, are morphologically distinctive, and are thus easily identifiable in air sampling surveys (63, 197). Both spore and cap extracts of *Ganoderma applanatum* were skin test reactive, and spore counts correlated generally with symptoms in studies done in New Zealand (64). *Ganoderma* sp. spores were also the most prevalent single spore type in Ontario, Canada, and studies with extracts detected high rates of skin-test reactivity there (35, 197). Two patients challenged with *Ganoderma lucidum* reacted positively in a bronchoprovocation study (117). The allergenicity of *Ganoderma* spp. has been studied by more laboratories than has that of any other basidiomycete. Despite the fact that several extracts are reasonably well characterized, no allergens have yet been isolated. SDS-PAGE immunoblots of *Ganoderma meredithae* spore and cap extracts with serum samples from six individuals who were positive by both RAST and skin test revealed 10 allergens (14 to >66 kDa) (75). Seven allergens occurred in both extracts, and three (66, 50, and 28 kDa) were unique to the cap. Isoelectric focusing immunoprints revealed a total of 16 allergens (pI <3.5 to 6.6); there were 8 each in both cap and spore (75). *G. applanatum* spore and fruiting body extracts tested by CLIE also demonstrated common antigens (39). In another study of *G. applanatum* spores, 14 antigens were detected by CIE, and immunoblots

revealed IgE-binding bands between 18 and 82 kDa (205). Thus, several laboratories have shown that extracts of *Ganoderma* spp. contain a number of allergens with a wide range of physicochemical properties.

Psilocybe cubensis. *Psilocybe cubensis* is allergenically one of the most significant species evaluated to date. *Psilocybe cubensis* induced more skin test reactions than other basidiomycetes when tested among clinic attendees in Europe and the United States (106, 107). In a RAST survey, it belonged to the group of species to which the greatest number of individuals had IgE (31). *Psilocybe cubensis* extracts showed the greatest inhibitory activity of all species studied by RAST inhibition by one group (139). SDS-PAGE immunoblot analyses of *Psilocybe cubensis* revealed 18 allergens (14 to 76 kDa) (65). On the basis of the prevalence of reactivity with 11 serum samples from patients who were skin test and RAST positive, the most important *Psilocybe cubensis* allergens were the 16-kDa (82% reactive), 35-kDa (100% reactive), and 76-kDa (91% reactive) proteins. Isoelectric focusing immunoprints revealed 13 allergens (pI >3.5 to 9.3). The resulting blots were screened with 15 serum samples, and the most reactive proteins were at pI 5.0 (80%), 5.6 (87%), 8.7 (80%), and 9.3 (100%). *Psilocybe cubensis* mycelium contains most, but not all, of the important spore allergens, although certain spore allergens (pI 4.0 and 5.7; 20 and 43 kDa) are not present in mycelial extracts (66). RAST inhibition studies indicated that common epitopes are present in the spore and mycelium extracts as well, but the spore extracts inhibited the RAST reaction better than the mycelial extract did. *Psilocybe cubensis* has proven to be a useful model for basidiospore allergen analysis. Reese et al. (158) have prepared MAbs to a 48-kDa allergen, which, for the first time, will allow structural analysis of a basidiomycete allergen. A cDNA clone that codes for the 16-kDa allergen has been isolated (81). The cDNA sequence and deduced amino acid sequence indicate that this allergen is homologous with cyclophilin, an abundant and widespread cytosolic isomerase.

Zygomycetes

Sugar and bread molds are familiar examples of zygomycetes. The key characteristic of the group is the sexual spore, the zygospore, formed by fusion of morphologically similar gametangia. Many species also produce abundant asexual sporangiospores that are dispersed by air currents and are of clinical importance as agents of opportunistic infections. Allergen characterization has been reported only for *Rhizopus nigricans* among the zygomycetes.

R. nigricans is a clinically important fungus abundant indoors, on damp walls in basement areas, and on stored, cooked foods. Extracts of the fungus have elicited positive skin test reactions in atopic subjects (55, 182). Thirty-one *R. nigricans* antigens have been recognized by CIE (188). Two allergens, Rhiz 3b (12 kDa; pI 4.8) and Rhiz 4b (14 kDa; pI 3.6) have been purified from crude extracts by ammonium sulfate precipitation, anion-exchange chromatography, and gel filtration. They are both glycoproteins, containing 6.75 and 6.54% protein, respectively. Each individual fraction was more potent in skin testing than the crude *R. nigricans* extract. Another recent study characterized antigens that react with IgG antibody which promise utility in the diagnosis of mucormycosis (219).

Yeasts

Yeasts, most of which are single celled, are a morphologic form of fungi. Various species within the Ascomycetes, Basidiomycetes, and Fungi Imperfecti have yeast forms (89). Yeasts have been reported to cause chronic urticaria (85) and respiratory allergic diseases (6, 7, 17, 94, 95, 170–174). Asthma from yeast contamination of cold-air home humidifiers has also been documented (183).

Candida albicans. Ten of 120 *Candida* species cause significant human infections. *Candida albicans* is the most frequently isolated pathogenic species (94), although it is found predominantly as normal flora of the gastrointestinal tract. Immediate skin test reactivity correlates with the occurrence of *Candida albicans* infection. The impairment of cell-mediated immunity may be associated with a disturbance of the IgE immunoregulatory response. IgE reactivity to *Candida albicans* allergens has been reported on several occasions, but the view that *Candida albicans* is a major inhalant allergen remains controversial.

In a study of asthmatic subjects, 71 of 149 were *Candida albicans* skin test positive (at 15 min). Fifty-five (77%) of the positive subjects had positive provocative inhalation challenge tests, and 43 (86%) of 50 of the inhalation-positive subjects had positive RASTs to *Candida albicans* antigens (7). Seventy-eight antigens were detected in *Candida albicans* by CIE. In SDS-PAGE immunoprints, several IgE-binding proteins (16 to 135 kDa) were evident. The IgE response was directed predominantly against two proteins (29 and 46 kDa) that proved to be the dominant allergens in six different *Candida albicans* strains (171, 174). Sera from 36% of the subjects reacted to a major cell wall carbohydrate, mannan (>70 kDa). Purified, fractionated mannan provoked immediate skin test reactions. This observation is controversial since many researchers assume that all allergens are protein. Since mannan is usually extracted as a mannoprotein containing 5 to 10% protein, some researchers feel that the allergenic epitopes are on the protein portion of the molecule. However, enzyme-linked immunosorbent assay (ELISA) inhibition with protease-treated and periodate-oxidized extracts showed an almost complete loss of IgE-binding activity due to periodate but only a partial reduction by proteases (46). This is a difficult issue to resolve, but support is strengthening for the premise that carbohydrate moieties can contain IgE epitopes.

Large-molecular-mass allergens (125 and 175 kDa) have been isolated by at least one other group (83). These large-molecular-mass allergens were not recovered by cell fractionation procedures that included zymosan treatment. Three other allergens (46, 43, and 37 kDa) were recovered, and homology was detected between these and *Saccharomyces cerevisiae* enolase, phosphoglycerate kinase, and aldolase, respectively (83). A 40-kDa *Candida albicans* allergen has also been cloned; sequence identity revealed 70% homology with alcohol dehydrogenase (73).

Malassezia furfur. *Malassezia furfur* (= *Pityrosporum ovale*) is a normal component of the dermal flora. *Malassezia furfur* (as *Pityrosporum orbiculare*) extracts induced positive skin tests and leukocyte histamine release in subjects with atopic dermatitis (84). SDS-PAGE immunoblots of *Malassezia furfur* extracts revealed several IgE-binding proteins. The dominant allergens were at 9, 15, 25, and 72 kDa (80). The 9- and 15-kDa components were mostly carbohydrate. Recent evidence also strongly implies that mannans from *Malassezia furfur* contain IgE epitopes (46, 48, 178). MAbs have been raised against a 67-kDa allergen of *Malassezia furfur* (as *Pityrosporum orbiculare*) (222). These MAbs do not cross-react with *Candida albicans* extracts and thus should prove useful to determine whether patients with atopic dermatitis are sensitized initially to *Malassezia furfur* or other yeasts.

Saccharomyces cerevisiae. *Saccharomyces cerevisiae* (baker's yeast) is an ascomycetous yeast. In a study of 47 subjects with suspected inhalant allergy to fungi, significant positive skin

tests (in 35 of 47) and RASTs (in 32 of 47) to *Saccharomyces cerevisiae* allergens were reported (17). Purified enolase from *Saccharomyces cerevisiae* has been shown to be the 51-kDa allergen in the extract (17). Although not reported as an aeroallergen, *Saccharomyces cerevisiae* may be an occupational inhalant allergen and may cross-react with *Candida albicans* (17).

Oomycetes

Most oomycetes are aquatic microfungi. The order Peronosporales is a group of oomycetes that are plant pathogens, and several of these produce copious numbers of airborne spores. Two members of this order, *Phytophthora infestans* and *Plasmopara viticola*, have been implicated as allergenic fungi. One early report provided circumstantial evidence for the induction of allergic asthma in three members of the same household following exposure to *Phytophthora infestans* on moldy potatoes (112). This study did not include controls, however. A more recent study using modern methodology described a case of occupational asthma related to exposure of a greenhouse worker to *Plasmopara viticola* spores (175). This study included nonatopic controls and in vitro analysis to characterize the allergens in the extracts. There is a potentially large population of agricultural and research workers that is exposed to *Plasmopara* spp. and other downy mildews. These fungi are obligate parasites, and, thus, allergen extracts are very difficult to produce; this will slow analysis of these allergens.

CROSS-REACTIVITY OF FUNGAL ALLERGENS

Cross-reactivity indicates the presence of epitopes shared by different fungal species. Cross-reactivity must be distinguished from parallel, independent sensitization to multiple fungal allergens (14). Skin tests will react positively in either case; therefore, shared epitopes are detected best with inhibition immunoassays. Thus, the degree of cross-reactivity between two fungi depends upon both the cross-reactive allergens and the serum (pool) used in the assay (14).

In general, shared epitopes have been described among species that are phylogenetically related. The degree of cross-reactivity between two species depends on the number of antigenic components that cross-react, the immunogenicity of the epitopes in a particular patient, and the method used to detect the antibodies. Experiments to detect cross-reactivity usually employ CLIE or competitive inhibition of methods such as RAST or immunoprint. The presence of cross-reactive epitopes among allergens is advantageous for the diagnostician because it reduces the number of antigens required in the panel of extracts used for testing. In theory, allergic reactions to any fungus in a group of highly cross-reactive species could be detected by testing with only a single representative species. Thus, a reasonably small panel of test reagents would be capable of detecting allergic reactions to a broad range of fungi. Establishing the extent and patterns of cross-reactivity is important because the use of multiple, cross-reacting allergens in skin tests or immunotherapy is redundant, and these tests should be avoided since there is a minimal risk associated with these procedures.

Cross-Reactivity among Ascomycete Anamorphs

Cross-reactivity among ascomycete anamorphs has been reviewed previously by Aukrust and Borch (14). Cross-reactivity has been demonstrated within *Aspergillus*, *Cladosporium*, and *Fusarium* spp. (87, 202, 203). RAST inhibition studies have indicated substantial cross-reactivity between *Aspergillus fumigatus*, one species of the *Aspergillus glaucus* group, and *Aspergillus flavus* (87). Unique allergenic determinants for each species have been detected as well. Culture filtrate antigens of three *Fusarium* spp. (*F. solani*, *F. equiseti*, and *F. moniliforme*) were each able to inhibit specific allergen bands of *F. solani* immunoblots (202). Cross-reactivity between *Cladosporium herbarum* and *Alternaria alternata* has been detected, but neither species cross-reacted with *Aspergillus fumigatus* by RAST or immunoblot inhibition (87, 198). Independently, CRIE and SDS-PAGE immunoblots have detected three or four antigens shared by *Cladosporium herbarum* and *Cladosporium cladosporoides* and cross-reactivity between these two fungi and *Alternaria alternata* (203, 204).

Inhibition studies employing RAST and *Alternaria alternata* extracts have been used to demonstrate cross-reactivity between *Alternaria alternata* and *Helminthosporium* spp., *Stemphylium* spp., *Curvularia* spp., and *Spondylocladium* (= *Stachylidium*) spp. (73). All species, except for *Stachylidium* spp., have teleomorphs (sexual stages) in the order Dothideales (Table 4). *Alternaria* and *Stemphylium* sp. extracts share many antigenic and allergenic determinants, including the major allergen Alt I (5). Significant cross-reactivity, including to Alt I, was also found between *Alternaria alternata* and *Ulocladium* spp. (56); these fungi may not be taxonomically distinct, however (33). In fact, *Stemphylium* spp. and *Ulocladium* spp. contained more Alt I than did the *Alternaria* sp. strain studied (4, 13). *Alternaria alternata* extracts significantly inhibited *Didymella exitialis* (Dothideales) extracts in a RAST (61), indicating significant cross-reactivity, but no inhibition was detected between *Alternaria alternata* and *E. nigrum* by ELISA with IgE-specific reagents. Significant cross-reactivity between the latter two genera could be detected with IgG-specific reagents, however (152).

One study reported some cross-reactivity between *F. solani* and *E. nigrum* (73). Both significantly inhibited *W. sebi* in RAST, but *W. sebi* only partially inhibited *Aspergillus fumigatus* (161). This finding suggests both shared and unique epitopes in *Aspergillus fumigatus*. An IgG ELISA inhibition study indicated that *B. cinerea* and *Aspergillus fumigatus* share epitopes (88).

Partial cross-reactivity among *B. cinerea*, *H. halodes*, and *Alternaria alternata* was determined by SDS-PAGE immunoblot and RAST inhibition (86). *F. solani* cross-reacts allergenically with *Penicillium notatum* and a species of the *Aspergillus glaucus* group (141), but *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *E. nigrum*, *Cladosporium herbarum* and *Alternaria alternata* do not inhibit *F. solani* in a RAST.

Thus, shared allergenic and antigenic epitopes appear common among ascomycete anamorphs. The full extent and range of this cross-reactivity remain unclear, although certainly the fungi with teleomorphs in the order Dothideales seem to cross-react extensively.

RAST cross-reactivity data should be interpreted cautiously. Fungal extracts are notoriously variable, and most researchers use only a single batch throughout an experiment. The absence of a particular allergen from an extract as a result of batch variability could erroneously suggest an absence of cross-reactivity. Thus, true allergen cross-reactivity studies would require comparable concentrations of the allergen in the two test extracts, permitting a rigorous analysis at the epitope level. This is clearly impossible at the present stage of allergen characterization but should soon be feasible as the number of available MAbs and recombinant allergens increases.

Cross-Reactivity among Basidiomycetes

High levels of cross-reactivity among a variety of basidiomycetes have been shown in RAST, immunoblot, and ELISA inhibition systems. In particular, significant cross-reactivity has been shown among *L. edodes*, *Agaricus bisporus*, and *Pleurotus ostreatus* spore allergens (168).

In a RAST inhibition investigation comparing spore extracts from *Amanita muscaria*, *Armillaria tabescens*, *Calvatia cyathiformis*, *Coprinus quadrifidus*, *G. lucidum*, *Pisolithus tinctorius*, *Pleurotus ostreatus*, *Psilocybe cubensis*, and *Scleroderma* spp., *Psilocybe cubensis* and *Pleurotus ostreatus* were the most potent inhibitors and *Pisolithus tinctorius* and *Scleroderma* spp. were the least (139). Cross-reactivity among six basidiomycete species was also assessed by isoelectric focusing and IgE immunoprint inhibition. Several allergens from *Coprinus quadrifidus*, *Calvatia cyathiformis*, *Pleurotus ostreatus*, and *Psilocybe cubensis* were cross-reactive (44). *G. meredithae* and *Pisolithus tinctorinus* did not show significant cross-reactivity with the other four species. The patterns described in these two studies broadly follow taxonomic lines. The members of the order Agaricales appear to contain important common allergens that occur in one of two puffball groups included. *Ganoderma* spp. and the *Pisolithus-Scleroderma* puffball order are considered taxonomically distinct, and they also appear to share fewer allergen epitopes.

Cross-Reactivity between Basidiomycetes and Ascomycete Anamorphs

It is not unusual for subjects who are sensitized to conidial fungi (Fungi Imperfecti) to also react to basidiomycetes. As discussed above, both skin test and RAST results may be positive as a result of either cross-reactivity or multiple, independent allergies; competitive inhibition assays are needed to distinguish the two. However, few such studies have been conducted, and only selected species of these fungi have been used. CLIE detected no antigenic cross-reactivity among *Cladosporium herbarum*, *Cantharellus cibarius*, and *Coprinus commatus* (162). Another CIE study showed no cross-reactivity among *Cladosporium cladosporoides*, *Cladosporium herbarum*, and *Alternaria alternata* (203).

Interestingly, results of RAST inhibition studies have varied. At one extreme, an *Alternaria alternata* RAST was not inhibited by any basidiomycete tested (*Calvatia cyathiformis*, *Armillaria tabescens*, *Pleurotus ostreatus*, or *Psilocybe cubensis*) (138). *F. solani* was inhibited only marginally by these same species, but a RAST with a species of the *Aspergillus glaucus* group was inhibited nearly 60% by *Psilocybe cubensis* and approximately 30% by *Calvatia cyathiformis* (138). *Armillaria tabescens* was inhibited 53, 38, and 22% by *Aspergillus fumigatus*, *E. nigrum*, and *Penicillium notatum*, respectively (138). Anamorphic ascomycetes only minimally inhibited *Calvatia cyathiformis* (138) and *Pleurotus ostreatus* RASTs (215).

These studies suggest that cross-reactivity between basidiomycetes and the ascomycete anamorphs tested is minimal or variable. This implies that panels of extracts for a RAST or skin test diagnosis of fungal allergy should include representative species from both fungal groups, since patients may be sensitive to one group but not the other.

Cross-Reactivity among Other Fungi

Airborne and dermatophytic fungi have been assumed to be cross-reactive, but there is no evidence of shared allergens (85). Allergenic determinants common to *Saccharomyces cerevisiae* and *Candida albicans* have been demonstrated by RAST inhibition (17). Antigenic (IgE) cross-reactivity and skin test correlations were shown among 8 of 10 *Candida* species but not with species of six other yeast genera (95). Marked cross-reactivity has also been demonstrated among the glycoprotein allergens of *Malassezia furfur* (as *Pityrosporum ovale*) and *Candida albicans* (47, 96, 169). Serological studies indicate common and unique antigens in *Cryptococcus* spp. (*Cryptococcus diffluens* and *Cryptococcus albidus*), *Candida* spp. (*Candida curvata* and *Candida humicola*), and *Trichosporon* spp. (176). Cross-absorption tests have revealed that *Cryptococcus neoformans* and *Trichosporon cutaneum*, (both members of the order Cryptococcaceae) are cross-reactive; *Trichosporon cutaneum* was only partially absorbed by *Cryptococcus neoformans*, while *Cryptococcus neoformans* was completely absorbed by *Trichosporon cutaneum* (181). Serological tests also indicated cross reactivity within *Trichophyton* spp. (*Trichophyton rubrum*, *Trichophyton tonsurans*, and *Trichophyton mentagrophytes*) but not among *Alternaria* spp. (43).

Shared epitopes do occur within several fungal groups (Dothidiales and Agaricales), but too few species have been examined to determine the limits of any general patterns. The cross-reactivity of rust and smut allergen epitopes is perhaps the largest remaining completely uncharted area of fungal allergens. This is in great need of study because of the prevalence of airborne rust and smut spores. A better understanding of fungal allergen cross-reactivity is very important clinically since identification of cross-reactive species reduces the number of extracts needed in a diagnostic panel without reducing the diagnostic value of the panel.

PURIFIED FUNGAL ALLERGENS

Purification

Major allergens are those to which the majority of allergic subjects react. All currently purified allergens are proteins or glycoproteins, although there are reports of carbohydrate allergens (97). Traditionally, allergens have been purified by standard liquid chromatography; recently, high-performance liquid chromatography (HPLC), fast protein liquid chromatography and preparative electrophoresis have also been used.

In the past, the potency and reactivity of fractionated or purified allergen were assessed by RAST inhibition or CRIE. While potency is still measured by RAST inhibition, immunoprinting and HPLC procedures are used more commonly to assess purity and determine physicochemical properties. The fungal allergens purified to date are listed in Table 3. This list should expand rapidly as fungal allergen genes are now being cloned and recombinant allergens are being produced.

MAbs

MAbs allow the isolation, characterization, and precise identification of fungal allergens (108). A significant advantage of hybridoma technology is that MAbs can be produced against allergens that are present in mixtures of antigens. This obviates the need for allergen purification but requires more extensive screening of clones. Once obtained, MAbs may be used to purify allergens by affinity chromatography (34). Theoretically, MAbs have unlimited availability, can be stored easily, and provide precise and sensitive tools to test allergen structures and standardize allergen preparations. Currently, MAbs are being used to develop immunoassays for quantitating fungal allergens in environmental samples, and MAbs are also being used to define specific epitopes. The application of MAb technology will probably be most useful in standardization of ex-

tracts. At present, human sera containing allergen-specific antibodies are still used in many of these assays. Complications arise because of the inherent variability of the human antibody response. Since MAbs are homogeneous, they will greatly reduce the variability of these immunoassays.

Test Methods for Purified Extracts

The simplest and most direct method of determining the allergenic activity of an extract is skin testing of allergic subjects. To evaluate nonspecific reactions, atopic individuals who are not sensitive to the test allergen must be included for study. Ideally, the investigator would prefer to have an in vitro assay for testing purified extracts in which test conditions could be controlled more tightly. In vitro testing is a very active research area. The RAST is the most widely used assay. The RAST and RAST inhibition are excellent methods to monitor purification procedures. RAST inhibition provides a quantitative estimate of the allergenic activity of a purified sample, but one cannot determine qualitatively which allergen has been detected. CRIE is an elegant but now less commonly used technique for allergen extract analysis. Direct challenges of sensitized subjects with small doses of extracts can confirm biological activity and allow estimates of the proportion of allergenicity that is due to the single, purified allergen. Gel electrophoresis techniques, including isoelectric focusing and SDS-PAGE, are very sensitive assays that are now widely used to assess allergen purity. Blots of gels can be probed with polyclonal sera to detect any contaminating allergen moieties. These methods have been used traditionally to characterize allergens purified from crude extracts. The rapidly increasing number of cloned allergen genes will allow production of large quantities of recombinant allergen. Recombinant proteins can be produced as a fusion with other proteins (or peptides) that facilitate purification, e.g., with MAb affinity techniques.

Standardization of Extracts

Previous reviews discuss the background and problems of allergen standardization (16, 30, 148, 149). More than 200 fungal extracts, produced by 12 manufacturers, are commercially available in the United States. None is standardized, and all are mixtures of multiple antigenic and allergenic components. Standardization must be established among extracts to ensure their consistent composition and activity. Skin tests and/or in vitro procedures may be the basis for standardization but must permit the determination of biologic potency of extracts from a variety of source materials and manufacturers.

Skin testing is a sensitive measure of the potency of an extract, but the responses of different populations to a selected allergen may vary and the reaction observed in a single individual may not always appear the same. These problems are a significant disadvantage of skin testing. In addition, skin testing reveals nothing about individual allergens in complex mixtures such as crude fungal extracts.

Efforts at standardization will benefit greatly from the use of MAbs (34, 108). Historically, one of the greatest needs for achieving standardization was a source of homogeneous antibodies to well-defined allergens. Sera from humans or animals with allergen-specific antibodies are always of limited supply, and animal antisera may not recognize the same epitopes as those of humans. Polyclonal antisera with the appropriate specifications are also difficult to produce in the large volumes required for standardization efforts. The use of MAbs can overcome these problems; they can be exquisitely specific and are available in large quantities, and with proper safeguards, cells that produce them are virtually immortal.

Political considerations further complicate allergen standardization. Regulatory agencies in different countries do not coordinate standardization procedures, and regulatory requirements vary considerably. At present, there are three independent systems for the expression of extract potency. Two systems, biological units and allergy units (AU), are based on skin test reactivity. The biological unit system originated in Scandinavia and is widely accepted in Europe. It compares the skin test reactivity of the test extract with the reactivity of histamine chloride (1 mg/ml) (1, 48). The allergy unit system was developed by the Food and Drug Administration in the United States and uses intradermal skin testing with threefold dilutions of extract. The intradermal end point is expressed as the number of threefold dilutions required to dilute the extract such that it will produce a 50-mm-wide erythematous reaction (200). International units, the unit of measure of the third system, are used for international standard preparations (ISPs). This system was established by the Subcommittee for Allergen Standardization of the International Union of Immunologic Societies and conforms to the requirements of the World Health Organization. The international unit is an arbitrary measure assigned to an ISP. Extracts compared with the ISP can be assigned a given number of international units on the basis of the relative potency of the extract and the ISP. Although in vitro quantitation of extracts by a RAST is more practical, the superiority of standardized extracts must still be demonstrated by their usefulness for skin tests. At present, no standardized fungal extracts are available. The only candidate, a standardized *Alternaria alternata* extract, has been tested in an international multicenter collaborative study but awaits World Health Organization acceptance (68). In spite of the lack of standardization, most companies monitor extract manufacturing procedures carefully and verify quality by RAST inhibition and other procedures (148).

CONCLUSIONS AND FUTURE DIRECTIONS

Relatively few allergens have been well characterized at the molecular level. Fel d 1, the major cat allergen, and Der p 1, the major mite allergen, are arguably the best studied of all allergens. However, the complete amino acid and DNA sequences of these allergens were reported in 1991 and 1988, respectively. Such structural analyses of fungal allergens are now becoming possible as genes for fungal allergens are being cloned.

MAbs are now being used with great success to analyze allergen structure. These analyses will contribute substantively to the identification and mapping of allergenic epitopes, which will in turn greatly improve standardization of extracts. There is also considerable interest in analyzing the T-cell epitopes of allergens. Although little is known as yet, the recognition of functional T-cell subsets (Th1 and Th2) in humans and of the role of these subsets in allergy should serve to increase research efforts in this area.

Our understanding of fungal allergy and allergens has long been plagued by the complexity and variability of fungal extracts and the far larger numbers of different fungal propagules than pollens in air samples. This problem may become less daunting after the extent of cross-reactivity is determined and the allergens requiring study are delimited. Once the DNA sequence of a major allergen is determined, other species that contain the gene may be identified by synthesis of oligonucleotide probes and use of genomic Southern blots or PCR amplification of gene regions of interest for sequence analysis.

Molecular cloning and DNA sequencing of allergen genes have been applied only recently to allergen research. In 1991,

a partial amino acid sequence was determined for the major allergen of *Aspergillus fumigatus* (10). The sequence of Asp f 1 cDNA was reported in 1992 (130), and the transcription of the Asp f 1 gene was shown to be restricted to *Aspergillus fumigatus* and *Aspergillus restrictus* and not to four other *Aspergillus* spp. (9). Also in 1992, partial cDNA sequences for Alt a 1, the major *Alternaria alternata* allergen, were presented by two groups (28, 125). In 1994, however, cloned sequences of five allergens were presented in abstracts. A particularly interesting product of this research will be the determination of the degrees of homology or functional similarity, if any, of the allergens. Such studies will enable full analysis of allergens at the structural and epitope (both B- and T-cell) levels. An understanding of allergen-specific T-cell responses may contribute to epitope structure studies as well, since allergen-specific T-cell clones can now be isolated, allowing the cytokine release pattern evoked by exposure to a single allergen or peptide fragment to be assessed in vitro.

Given the large number of allergenic fungi and the prevalence of fungal allergy, few studies have attempted to determine the clinical relevance of exposure to fungal allergens. The most extensively characterized species are the conidial ascomycetes, such as *Alternaria alternata*, *Aspergillus fumigatus*, and *Cladosporium herbarum*. New, more sophisticated immunologic techniques have increased the rate at which allergens from the aforementioned fungi and from the basidiomycetes have been distinguished. The efficacy of immunotherapy with the currently available partially standardized extracts has been demonstrated. To achieve the most reliable standardization (based on epitopes), a thorough elucidation of allergen structure at the molecular level is required. Only extracts developed in this way will provide definitive reagents for immunotherapy. Further characterization of known fungal allergens by use of a molecular genetic approach and characterization of allergens produced by additional fungal species are clearly warranted and should result in improved extract quality.

Immunochemical techniques to quantify airborne allergens may provide a background for correlating patient symptoms and spore exposure. Efforts are needed to identify important allergens in particular geographic areas, and clinicians should be aware of the local allergen load in providing care for patients when allergen avoidance is impractical. Candidates for fungal immunotherapy include patients with (i) documented symptoms (rhinitis or asthma) that occur during periods of elevated airborne spore counts; (ii) IgE to well-characterized fungal allergens, as shown by skin test, RAST, and ELISA, etc.; and (iii) symptoms that cannot be controlled by conventional pharmacotherapy or avoidance. These guidelines are discussed fully elsewhere (164). Two major impediments to treating mold allergy are the lack of standardized extracts and the inability to clearly demonstrate the efficacy of immunotherapy for species other than *Alternaria* spp. The application of molecular biology to these problems holds great promise. MAbs have already enhanced the isolation and characterization of these allergens. PCR-based DNA sequence analysis will allow the rapid comparison of specific allergen genes from many species to help establish the limits of allergenic cross-reactivity. Cloning of allergen genes will facilitate epitope identification by allowing the deletion of portions of expressed proteins. Finally, site-directed mutagenesis will permit the modification of epitopes and produce altered allergens that may provide new, safer, and more effective treatment for mold allergy than that which currently exists.

The most pressing need for fungal allergen research is a panel of potent, well-defined allergenic extracts with known patterns of cross-reactivity. Such a panel would permit definitive immunotherapy trials and could provide an accurate determination of the prevalence of fungal allergy. Defined allergens are also useful to generate MAbs which can be used to measure environmental allergen concentrations. In this way, threshold exposures can be determined, and the efficacy of allergen reduction strategies can be documented. Thus, the field of fungal allergy research, diagnosis, and treatment is now poised for rapid development as cloning techniques provide the genes and recombinant allergens for analysis.

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